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Three genes encode 3-hydroxy-3-methylglutaryl-coenzyme A reductase in *Hevea brasiliensis*: *hmg1* and *hmg3* are differentially expressed

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Abstract

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) catalyses an important step in isoprenoid biosynthesis in plants. In *Hevea brasiliensis*, HMGR is encoded by a small gene family comprised of three members, *hmg1*, *hmg2* and *hmg3*. We have previously described *hmg1* and *hmg2* (Plant Mol Biol 16: 567–577, 1991). Here we report the isolation and characterization of *hmg3* genomic and cDNA clones. In comparison to *hmg1* which is more highly expressed in laticifers than in leaves, the level of *hmg3* mRNA level is equally abundant in laticifers and leaves. *In situ* hybridization experiments showed that the expression of *hmg3* is not cell-type specific while *hmg1* is expressed predominantly in the laticifers. Primer-extension experiments using laticifer RNA showed that *hmg1* is induced by ethylene while *hmg3* expression remains constitutive. The *hmg3* promoter, like the promoters of most housekeeping genes, lacks a TATA box. Our results suggest that *hmg1* is likely to encode the enzyme involved in rubber biosynthesis while *hmg3* is possibly involved in isoprenoid biosynthesis of a housekeeping nature.

Introduction

The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) catalyses the synthesis of mevalonate from HMG-CoA. Mevalonate is converted to isopentenyl pyrophosphate (IPP) which acts as precursor to a wide range of isoprenoid compounds in different organisms. In plants these include growth regulators (abscisic acid, gibberellins and cytokinins), photosynthetic pigments (chlorophylls tocopherols, plastoquinone, carotenoids), mitochondrial electron transfer chain components (ubiquinone and haem *a* of cytochrome oxidase), dolichol, phytoalexins and

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers M74798 (*Hevea brasiliensis hmg3* gene), M74799 (*Hevea brasiliensis hmg3* promoter) and M74800 (*Hevea brasiliensis hmg3* cDNA)

natural rubber. The diversity of these isoprenoid compounds and the occurrence of two *hmg* genes in *Arabidopsis* [8], *Hevea* [10] and potato [34] seem to suggest that multiple pathways exist for the biosynthesis of IPP. The biosynthesis of different isoprenoid compounds like chlorophylls and natural rubber which occurs in specialized cell types further suggests that *hmg* genes may be expressed differentially. Recently potato *hmg* genes have been reported to be regulated differentially. Yang *et al.* [34] used a tomato *hmg* probe to show the presence of two *hmg* genes in potato, one isogene is induced by wounding while the other is induced by pathogen challenge.

Hevea brasiliensis is commercially grown in South-East Asia for the production of natural rubber. This unique isoprenoid compound, *cis*-1,4-polyisoprene (M_r $4x10^6$) [32], is present in latex, a milky fluid stored in specialized plant cells called laticifers, which are interspersed with the phloem cells. The laticifers contain all the normal cell constituents plus rubber particles and characteristic organelles (lutoids and Frey-Wyssling particles). Hevea HMGR has been implicated to be a membrane-bound enzyme [15, 29, 30] present in the pelleted portion of centrifuged latex, that requires NADPH and thiol compounds for its activity [31]. Recently, we have identified two classes of *hmg* cDNAs from *H. brasiliensis*, *hmg1* and *hmg2* [10]. Comparison of the two classes shows 86% nucleotide sequence homology and 95% amino acid homology [10]. The high homologies encountered are related to the isolation of partial *hmg2* cDNA clones which encompass the HMGR conserved region. Further characterization of *hmg2* has been hindered by the unavailability of a full-length cDNA clone. We are interested to investigate the expression and regulation of the different *hmg* genes in *Hevea* and to determine if the isoprenoid pathway leading to rubber biosynthesis is distinct from other isoprenoid pathways since this plant is unique in producing rubber.

In the present paper, we describe the isolation and characterization of a third class of genes encoding HMGR in *Hevea*, *hmg3*. We show that *hmg1* and *hmg3* are differentially expressed in laticifer and leaf. Our results indicate that *hmg1* is involved in rubber biosynthesis whereas *hmg3* is involved in isoprenoid biosynthesis of a housekeeping nature.

Materials and methods

Plant material

Hevea brasiliensis RRIM600 plants were grown in pots under natural conditions (12 h light/12 h dark cycle at 25–34 °C). Genomic DNA was obtained from young leaves and purified by CsCl centrifugation [23]. Total cell RNA was isolated from leaves of 1- to 3-year-old plants as described [24]. Laticifer RNA was prepared from the latex of field-tapped trees [17].

Ethephon treatment of *Hevea* trees was carried out by applying 5% ethephon on the tree trunk to 2 cm of scraped bark below the cut and at the groove on alternate months. The tree was tapped every six days by making an incision in the bark half a spiral around the tree trunk in order to sever the laticifers. The latex was collected at the bottom of the cut. Control trees which were not treated with ethephon were similarly tapped.

Screening of cDNA and genomic libraries

A *Hevea* λDASH genomic library was screened in duplicate by *in situ* plaque hybridization using the 1.3 kb *hmg1* cDNA (positions 857 to 2204 of the *hmg1* cDNA in Fig. 1) [10]. A *Hevea* oligo (dT) leaf cDNA library [10] was screened for *hmg3* cDNA clones by using a *hmg3*-specific 1.2 kb *Eco* RV-*Eco* RI genomic fragment, derived from the 3′ end of an HMGR3 genomic clone. The *Eco* RV site of the 1.2 kb fragment corresponds to nucleotide position 2009 of the *hmg3* cDNA in Fig. 1. The 1.2 kb fragment basically consists of positions 2009 to 2351 of the *hmg3* cDNA in Fig. 1 and 0.8 kb of 3′-end untranscribed region. Putative positive clones were purified and their DNAs isolated by CsCl gradient centrifugation [23].

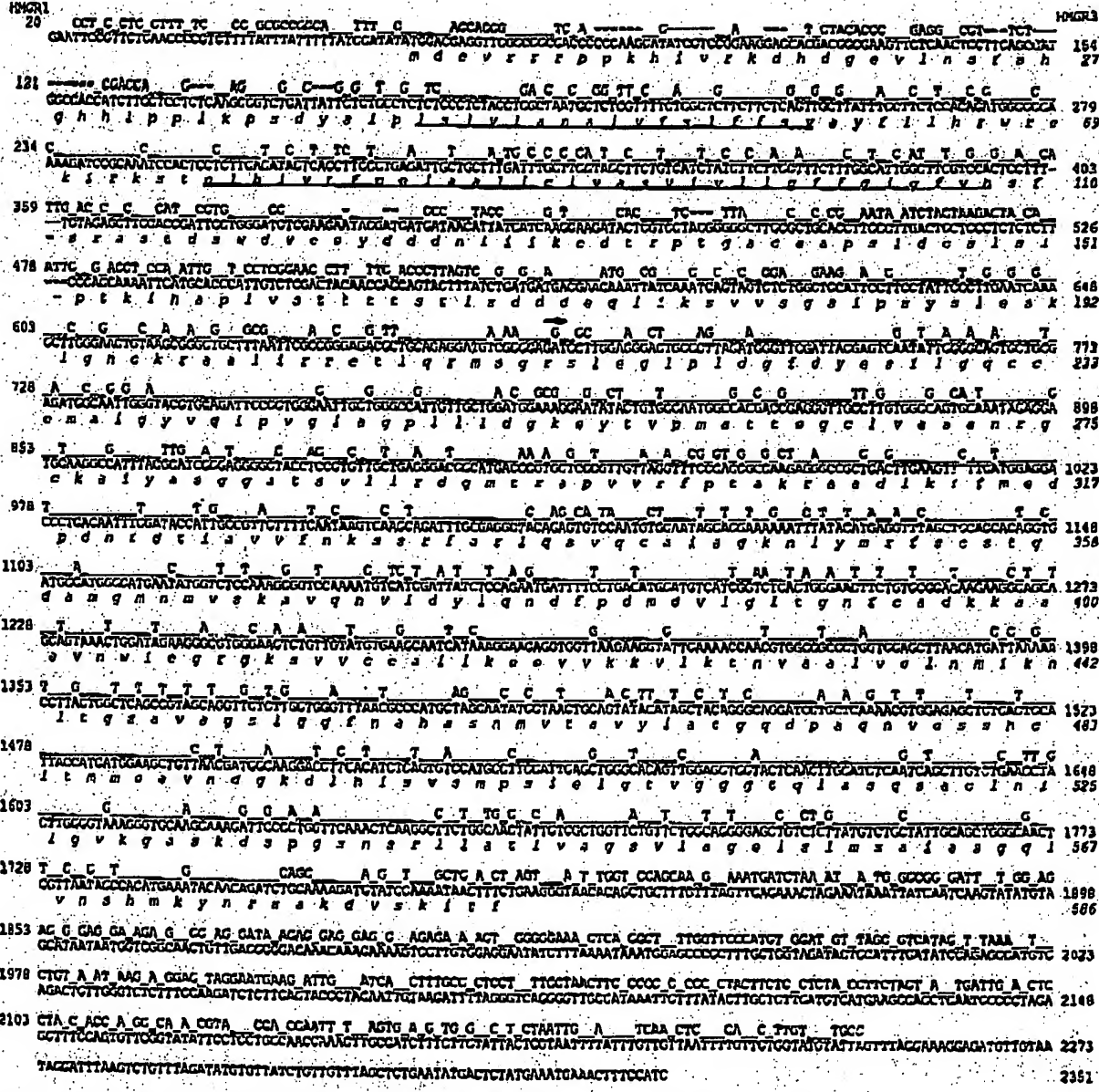

Fig. 1. Comparison of nucleotide sequences of *hmg1* and *hmg3* from *Hevea brasiliensis*. The nucleotide sequence of *hmg1* cDNA is shown above that of *hmg3* cDNA. Only non-identical nucleotides in the *hmg1* cDNA sequence are shown and mismatches are indicated. The nucleotides of each cDNA are numbered with respect to the transcription start site as mapped by primer extension. The HMGR3 deduced amino acid sequence is shown below its nucleotide sequence and the residues are numbered with respect to the ATG start codon. The nucleotide and deduced amino acid sequences of *hmg3* cDNA are numbered on the right of the figure while the nucleotide sequence of *hmg1* cDNA is numbered on the left. The 5′ border of the 3.5 kb *hmg3* genomic clone corresponds to nucleotide 711 of the *hmg3* cDNA and is denoted by an arrow. The potential transmembrane domains of *hmg3* are underlined.

DNA sequence analysis

DNA fragments containing the sequence of interest were subcloned into M13mp18 [35] for DNA sequencing [28]. The sequences of both strands were determined using synthetic oligonucleotide primers which were ca. 0.4 kb apart.

Genomic Southern blot analysis

For genomic Southern analysis, high-molecular-weight DNA (20 μg) was digested with various restriction endonucleases, separated by electrophoresis in 0.7% agarose gels and blotted onto Hybond N (Amersham, UK) filters according to standard procedures [23]. Filters were prehybridized in 6× SSC, 0.5% SDS, 10% dextran sulphate and 100 μg/ml single-stranded salmon sperm DNA, then hybridized with labelled probe at 65 °C for 16 h under the same conditions.

Northern blot analysis

Twenty μg of total RNA were denatured at 50 °C in the presence of glyoxal, separated by electrophoresis in 1.5% agarose gel and blotted onto Hybond-N (Amersham) filters. The conditions of blotting, prehybridization and hybridization were as recommended by the manufacturer.

Primer extension analysis

To map the 5′ end of the *hmg3* mRNA a ^{32}P-end-labelled oligomer (5′-TCCTTCCGGACG-ATATGCTTGGGGGGTC-3′) complementary to the cDNA sequence from positions +93 to +120 (Fig. 1) was hybridized to 50 μg of total RNA from leaf and laticifer. Extension with MMV reverse transcriptase was carried out following standard protocols [2].

To compare the expression of *hmg1* and *hmg3* in response to ethylene the *hmg3* oligomer described above and a ^{32}P-end-labelled *hmg1*-specific oligomer (5′-GCATGCTTTCGGTGG-TGGAGCCGGCCGGTGGTGTCCATGT-3′) complementary to the *hmg3* cDNA sequence from positions +59 to +98 (Fig. 1) were hybridized to leaf RNA, laticifer RNA from ethephon-treated or control trees. Fifty μg RNA was used in each case. Extension with MMV reverse transcriptase was carried out as described [2].

In situ *hybridization experiments*

In situ hybridization experiments were carried out following conditions described by Cox and Goldberg [12]. A 0.1 kb *Eco* RI-*Sph* I fragment containing the 5′ end of *hmg1* cDNA (positions 20 to 97 in Fig. 1) and a 0.4 kb *Eco* RI-*Xba* I fragment containing the 5′ end of *hmg3* cDNA (positions 30 to 404 in Fig. 1) were cloned into Bluescript SK-(Stratagene) in which the T3 and T7 RNA polymerase promoters are in opposite orientations. ^{35}S-labelled antisense and sense RNA probes were synthesized *in vitro* and hybridized with petiole sections overnight at 40 °C. Sections were washed following procedures described previously [12]. Slides were coated with nuclear track emulsion (Kodak NTB-2), and were developed after 2 weeks.

Results

Isolation and DNA sequence analysis of Hevea hmg3 *genomic and cDNA clones*

We used the *hmg1* cDNA to screen a λDASH *Hevea* genomic library for *hmg1* genomic clones and obtained several putative clones. Upon further characterization by restriction analysis we found that one particular clone was different from the *hmg1* genomic clone.This clone had a 3.5 kb *Eco* RI fragment that hybridized to the *hmg1* cDNA but its nucleotide sequence was different from those of *hmg1* or *hmg2*. The 5′ end of the 3.5 kb *Eco* RI fragment overlapped with the coding region starting from position 665 of the *hmg1* cDNA. Comparison of these two sequences showed 71.4% nucleotide sequence homology (Fig. 1). These results indicated that the 3.5 kb *Eco* RI fragment encodes a different *hmg* gene which we have designated *hmg3*. We found that the 5′ end of the 3.5 kb *Eco* RI fragment was the site where *Sau* 3A was ligated to the *Bam* HI site of vector λDASH and therefore it was not possible to retrieve the 5′ end of *hmg3* from the same genomic clone.

To isolate the *hmg3* cDNA clones, a *hmg3*-

specific 1.2 kb *Eco* RV-*Eco* RI genomic fragment (see Materials and methods) was used to screen a *Hevea* leaf cDNA library. Several putative clones were isolated and sequenced. Figure 1 shows the sequence of *hmg3* cDNA. The *hmg3* cDNA consists of 36 bp of 5′-untranslated region, 1758 bp of coding region, 520 bp of 3′-untranslated region and a poly(A) tail. Comparison of the nucleotide sequences of the *hmg3* cDNA and genomic clone revealed that the reading frame in the genomic DNA is interrupted by three introns of 369 bases, 247 bases and 422 bases, occurring after nucleotide positions 1062, 1244 and 1591, respectively, on the *hmg3* cDNA (Fig. 1). These three introns that interrupt the *hmg3*-coding sequence occur in the same positions as in *Hevea hmg1* [10] and *Arabidopsis hmg* [8, 19]. Nucleotide sequence comparison of *hmg3* and *hmg1* cDNAs shows 70% nucleotide sequence homology; the homology was reduced at the 5′ end and the 3′ end of the cDNA (Fig. 1).

Analysis of the predicted amino acid sequence of HMGR3

The amino acid sequence of *Hevea* HMGR3 deduced from the DNA sequence (Fig. 1) reveals an open reading frame of 586 amino acids which would encode a protein of M_r 62 978. In comparison, the *Hevea* HMGR1 polypeptide comprising of 575 amino acids (M_r 61 702) is slightly smaller in size while that of *Arabidopsis* HMGR (M_r 75 785) is larger [8, 10, 19]. Comparison of the HMGR3 polypeptide with that of HMGR1 shows 77% amino acid homology with high conservation at the carboxy terminus of the polypeptide (Fig. 2).

The hydrophobicity plot of HMGR3 does not significantly differ from those of HMGR1 and *Arabidopsis* HMGR (Fig. 3). Two hydrophobic domains are conserved in plant HMGRs while seven such domains occur in mammalian [20] and yeast HMGRs [5]. The two potential transmembrane domains as predicted following the method of Klein *et al.* [16] present in HMGR1 (amino acid residues 32–48 and 65–97) and *Arabidopsis* HMGR (amino acid residues 53-69 and 86–118) are also found in HMGR3 (amino acid residues 43–59 and 76–108) (Fig. 2). This is consistent with previous reports that plant HMGRs are membrane-bound [3, 14]. The seven transmembrane domains in hamster [6, 13, 20] and in yeast [33] are involved in anchoring the enzyme to the endoplasmic reticulum.

We note that although there is less amino acid

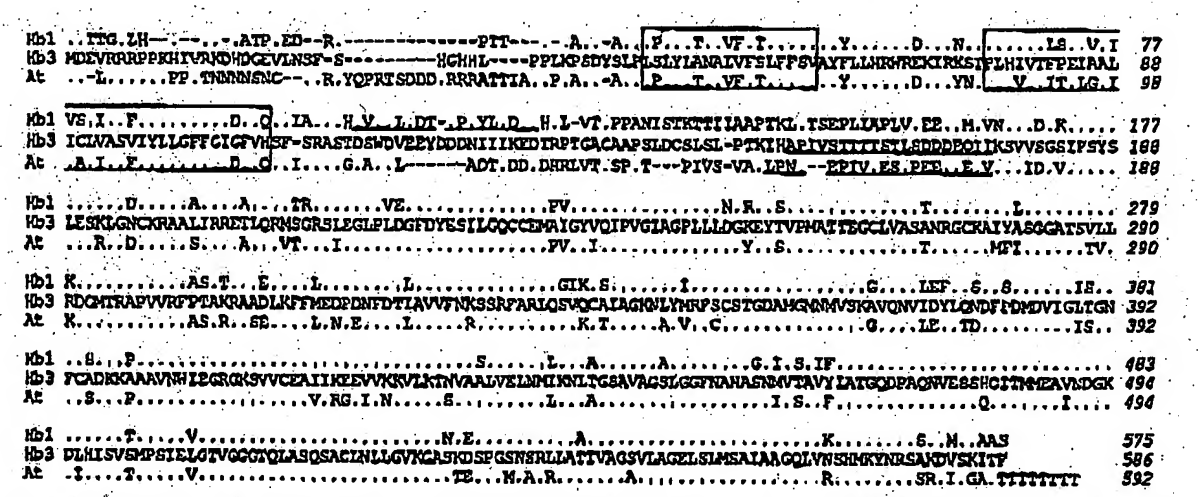

Fig. 2. Comparison of the predicted amino acid sequences of *Hevea* HMGR3 (Hb3), *Hevea* HMGR1 (Hb 1) [10] and *Arabidopsis* HMGR (AT) [19]. Positions of identity to HMGR3 are denoted by dots. The potential transmembrane domains are boxed and the PEST sequences underlined.

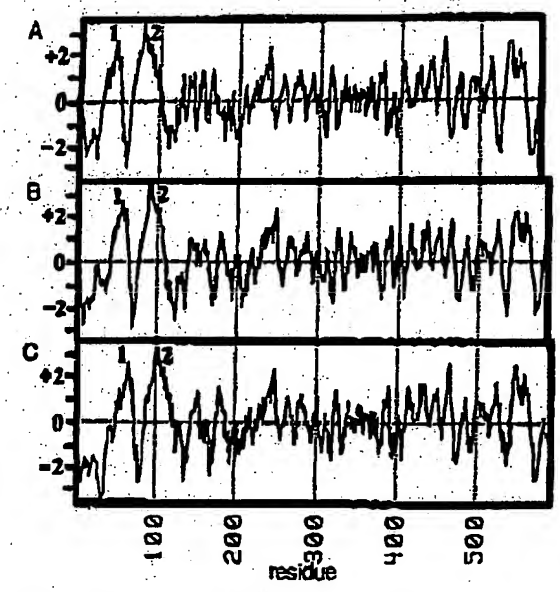

Fig. 3. Hydropathy plots of *Hevea* HMGR1 (A), *Hevea* HMGR3 (B) and *Arabidopsis* HMGR (C). The average hydrophobicity of each amino acid residue was calculated by the method of Kyte and Doolittle [18] over a window of nine amino acids and was plotted as a function of amino acid position. The two potential transmembrane domains are numbered.

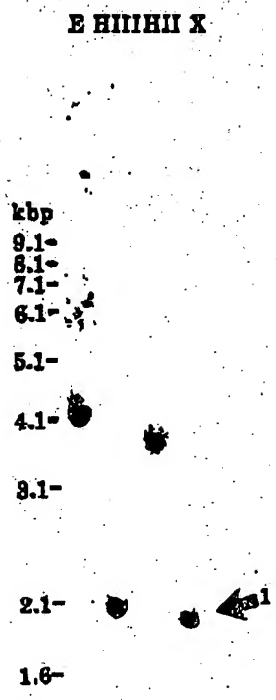

Fig. 4. Genomic Southern analysis to identify *hmg3* promoter-containing fragments. *Hevea* genomic DNA (20 μg) was digested with *Eco* RI (E), *Xba* I (X), *Hind* III (HIII) and *Hind* II (HII), separated by gel electrophoresis and blotted onto Hybond N (Amersham) membrane. The genomic blot was hybridized to a 5′-end *hmg3*-specific probe (0.4 kb *Eco* RI-*Xba* I fragment, positions 30 to 404 of the *hmg3* cDNA in Fig. 1). Arrow 1 refers to a 2 kb *Xba* I fragment which hybridized to this probe.

sequence conservation at the amino-termini of *Hevea* HMGR1, *Hevea* HMGR3 and *Arabidopsis* HMGR, the amino acids within the hydrophobic domains and those located in-between the two hydrophobic domains nevertheless remain conserved (Fig. 2). A PEST sequence [27] which is present downstream from the hydrophobic domains in *Hevea* HMGR1 (amino acid residues 106–122) and *Arabidopsis* HMGR (amino acid residues 157–176) is also found in HMGR3 (amino acid residues 157–176) (Fig. 2). It is striking that 80% of the first ten amino acid residues of *Hevea* HMGR3 and *Arabidopsis* HMGR are identical (Fig. 2).

Cloning the Hevea hmg3 *promoter*

Sequence comparison of the cDNA clones revealed that *hmg1* and *hmg3* are divergent in their 5′ ends (Fig. 1). A *hmg3*-specific probe consisting of a 0.4 kb *Eco* RI-*Xba* I fragment (positions 30 to 404 of the *hmg3* cDNA in Fig. 1) was used in genomic Southern analysis to identify bands which hybridize to the 5′ end of *hmg3* cDNA. Several such hybridizing bands were identified, one being a 2 kb *Xba* I genomic fragment (indicated by arrow 1 in Fig. 4). In order to make a λ library enriched for the *hmg3* promoter, *Xba* I-digested genomic DNA was sized by agarose gel electrophoresis and DNA fragments of about 2 kb were extracted and ligated to λGEM2 (Promega) which has a unique *Xba* I site. The resultant library was then screened with the 0.4 kb *Eco* RI-*Xba* I probe to identify clones bearing the *hmg3* promoter. One putative clone was shown by restriction analysis to contain a 2 kb *Xba* I fragment. DNA sequence analysis of this fragment yielded a sequence which had a 0.4 kb

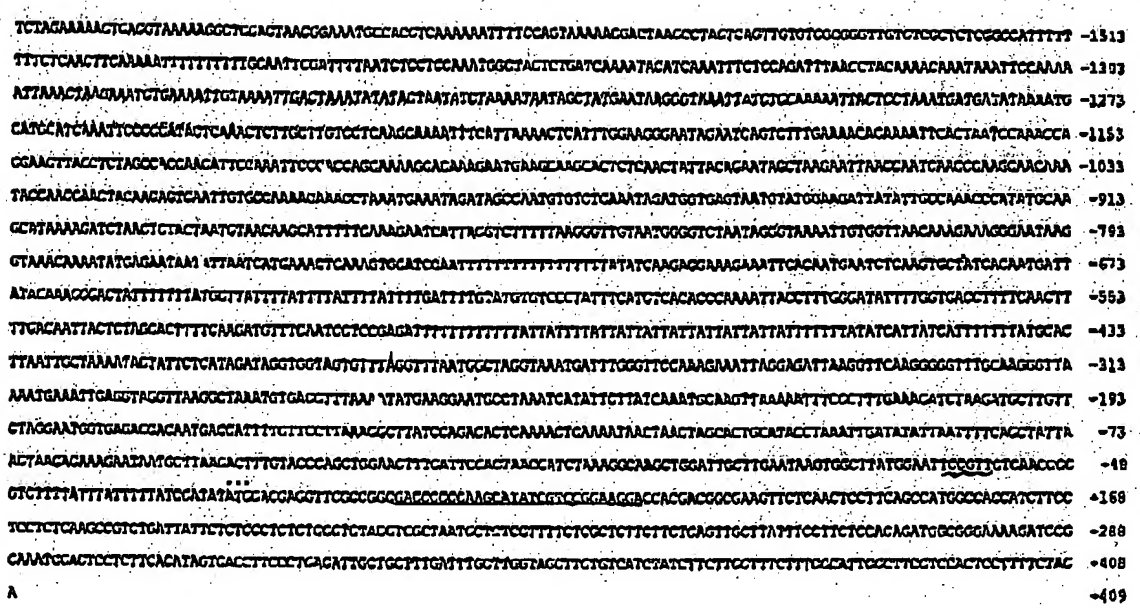

Fig. 5. Nucleotide sequence of a 2 kb *Xba* I fragment containing the *hmg3* promoter. The nucleotides are numbered with respect to the transcription start site (shown in bold) as mapped by primer-extension. The cDNA sequence at position + 93 to + 120 which is complementary to the primer used in primer-extension is underlined. The 5′ border of the *hmg3* cDNA is located at nucleotide + 30 and the ATG start codon of *HMGR3* is denoted by a dotted line above the sequence. The sequence of a putative downstream promoter element, 'TCCGTT' is denoted by a wavy line.

Eco RI-*Xba* I overlap with the *hmg3* cDNA. The sequence of the *hmg3* promoter is shown in Fig. 5.

Analysis of hmg3 *RNA*

When the *hmg3*-specific 0.4 kb *Eco* RI-*Xba* I probe was used in northern analysis it was shown that the *hmg3* transcript of 2.4 kb is present in both leaf and laticifer (Fig. 6). The size of the *hmg3* transcript predicted by northern analysis corresponds well to the size of the *hmg3* cDNA of 2.6 kb. In comparison, the *Hevea hmg1* transcript [10] and the *Arabidopsis hmg* transcript [8] are also 2.4 kb in size.

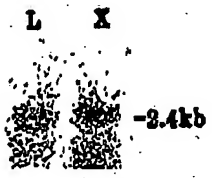

Fig. 6. Northern blot analysis. Total RNA (20 μg) from *Hevea* leaf (L) and laticifer (X) were hybridized to the 5′-end *hmg3*-specific cDNA (0.4 kb *Eco* RI-*Xba* I fragment, positions 30 to 404 of the *hmg3* cDNA in Fig. 1).

We used primer-extension analysis to identify the 5′ end of the *hmg3* mRNA (Fig. 7) and the location is shown in Fig. 5. When mRNAs from leaf and laticifer were compared we found that the *hmg3* mRNA is equally abundant in both tissues confirming the northern blot analysis (Fig. 6). Examination of the *hmg3* promoter sequences upstream of the transcription start site showed the absence of a typical TATA box (Fig. 5). This would not be surprising if *hmg3* were a housekeeping gene, as promoters of many housekeeping genes including hamster *hmg* [26] and human *hmg* [21] also a TATA box. However, the promoter of *Hevea hmg3*, unlike those of hamster *hmg* [26] and human *hmg* [21], do not contain GC-rich ('CCGCCC' or 'GGGCGG') hexanucleotide sequences. Moreover, multiple transcription start sites which are detected in hamster *hmg* [26] and human *hmg* [21] are not found in *Hevea hmg3*. Arkhipova and Ilyin [1] have reported a specific class of RNA polymerase II promoters

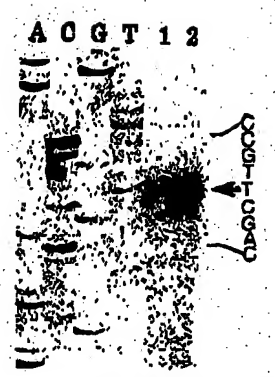

Fig. 7. Primer-extension analysis to map the 5′ end of the *hmg3* transcript. A ^{32}P-labelled primer (complementary to positions +93 to +120 of the *hmg3* cDNA in Fig. 5) was hybridized to 50 μg of leaf total RNA (1) and 50 μg of laticifer total RNA (2) from *Hevea*. This primer was also used to generate a dideoxy sequencing ladder (ACGT) wich was electrophoresed next to the extended product. The template used in this sequencing reaction was derived from a M13mp18 clone containing the 2 kb *Xba* I genomic fragment of the *hmg3* promoter for which the sequence is given in Fig. 5.

which lacks both TATA box and GC-rich regions and is characterised by a downstream promoter element 'TCAGTPy' located within 40 bp from the transcription start site. We have located a sequence 'TCCGTT' which shows homology to this consensus sequence at 33 bp from the transcription start site of *hmg3* (Fig. 5).

Three genes encode HMGR in Hevea brasiliensis

We have previously shown by genomic Southern analysis using *hmg1* and *hmg2* 3′-end-specific probes that *hmg1* and *hmg2* correspond to two different genes in the *Hevea* genome [10]. Comparison of the cDNA sequences of *hmg1* and *hmg3* has shown that their 3′-untranslated regions are divergent (Fig. 1). Therefore a 3′-end 0.4 kb *Ava* II-*Eco* RI fragment from *hmg1* and a 3′-end 1.2 kb *Eco* RV-*Eco* RI genomic fragment from *hmg3* were each used as discriminating probes in genomic Southern analysis. When the genomic blot was hybridized to the 3′-end probe of *hmg1*, a 2.6 kb hybridizing band (denoted by arrow 1 in Fig. 8A) was observed in the *Bam* HI digest. However, when the 3′-end probe of *hmg3*

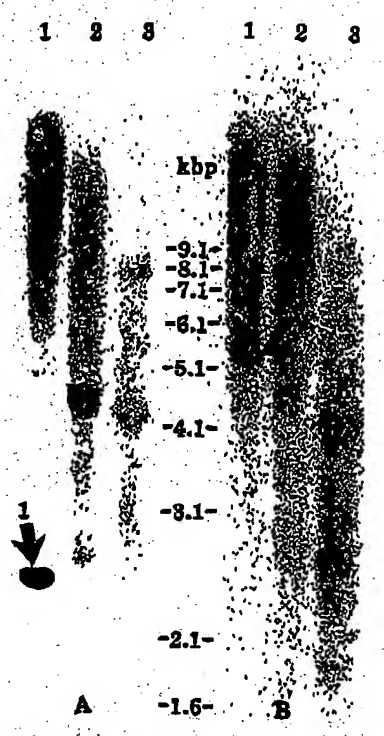

Fig. 8. Genomic Southern analysis using *hmg1*-specific and *hmg3*-specific probes. *Hevea* genomic DNA (20 μg) was digested with *Bam* HI (lanes 1), *Eco* RI (lanes 2) and *Hind* II (lanes 3), separated by gel electrophoresis, blotted onto Hybond N (Amersham) membrane and hybridized with the following ^{32}P-labelled DNA probes: A, *hmg1* cDNA (0.4 kb *Ava* II-*Eco* RI fragment, positions 1799 to 2204 of the *hmg1* cDNA in Fig. 1) and B, *hmg3* 3′-end genomic fragment (1.2 kb *Eco* RV-*Eco* RI fragment consisting of 0.4 kb DNA corresponding to positions 2009 to 2351 of the *hmg3* cDNA in Fig. 1 and 0.8 kb of the 3′-flanking region on the 3.5 kb genomic clone). Arrows 1 and 2 refer to the 2.6 kb hybridizing band and the 5.3 kb hybridizing band, respectively.

was used, a 5.3 kb band (denoted by arrow 2 in Fig. 8B) was seen in this digest. Similarly, in the *Eco* RI and *Hind* II digests, the set of band(s) hybridizing to the 3′-end probe of *hmg1* is distinct from that hybridizing to the 3′-end probe of *hmg3*. These results demonstrate that *hmg1* and *hmg3* are distinct genes.

hmg1 *and* hmg3 *are differentially expressed and are differentially regulated by ethylene*

The presence of three genes encoding HMGR in *Hevea brasiliensis* raises the question of their func-

tions, especially in relation to rubber biosynthesis. While the characterization of *hmg2* has been limited by the availability of only a partial cDNA, information of complete cDNA sequences of *hmg1* and *hmg3* has provided us tools for further experiments. We used specific primers in primer-extension analysis to confirm results from northern analysis that *hmg1* and *hmg3* are expressed differentially in laticifer and leaf. Primer extension analysis was carried out using a *hmg1*-specific primer together with a *hmg3*-specific primer to show that the *hmg3* mRNA is constitutively expressed in laticifer and leaf while *hmg1* mRNA is more abundant in laticifer than leaf (lanes 2 and 4 in Fig. 9). In the control in which only the *hmg1*-specific primer was used, the *hmg3* extension product was absent (lane 1 in Fig. 9).

We also examined the role of ethylene on HMGR expression. Ethephon (2-chloroethane phosphonic acid) which generates ethylene *in vivo* has been applied regularly, for decades, on the trunks of rubber trees to stimulate latex yield. The exact physiological and biochemical processes that result from this treatment are unclear [11]. Since HMGR is involved in rubber biosynthesis and *hmg1* appears to be more highly expressed in laticifer than leaf, we are particularly interested to study the role of ethylene on *hmg1* expression. When RNA obtained from laticifer of ethephon-treated trees was used in primer-extension analysis, we observed that the expression of *hmg1* is induced while *hmg3* remains constitutively expressed (lane 3 in Fig. 9). Ethylene-inducible *hmg1* is likely to encode the HMGR involved in rubber biosynthesis. An increase of this enzyme, if it is rate-limiting, will inadvertently lead to an increase in rubber biosynthesis and subsequently result in a higher latex yield. In hamster, HMGR has been shown to be the rate-limiting enzyme in cholesterol biosynthesis [7].

Fig. 9. Primer-extension analysis to show that *hmg1* and *hmg3* are differentially regulated by ethylene. ^{32}P-labelled *hmg1*-specific and *hmg3*-specific primers were both hybridized to 50 μg of laticifer total RNA from non-treated control trees (lane 2), 50 μg of laticifer total RNA from ethephon-treated trees (lane 3) and 50 μg leaf total RNA (lane 4) and extended with MMV reverse transcriptase. The extended products of *hmg3* and *hmg1* are marked respectively. In lane 1, only the *hmg1*-specific primer was used to hybridize 50 μg of laticifer total RNA from ethephon-treated trees. The *hmg1*-specific primer was used to generate a dideoxy sequencing ladder (ACGT) which was electrophoresed next to the extended products. The mapping of the 5′ end of the *hmg1* transcript has been previously reported [10].

Localization of the hmg1 *and* hmg3 *transcripts by* in situ *hybridization studies*

In situ hybridization experiments on petiole sections of the *Hevea* plant have shown that the *hmg1* mRNA is specific to laticifers while the *hmg3* mRNA appears to be distributed over all cells (Fig. 10). These results suggest that *hmg1* encodes the enzyme involved in *cis*-1,4-polyisoprene biosynthesis since the laticifers in *Hevea* are the sites for rubber biosynthesis. *hmg3*, which appears to be widely distributed over all cells, is likely to encode a housekeeping HMGR responsible for the biosynthesis of other plant isoprenoid compounds.

Discussion

We have isolated and characterized a third class of genomic and cDNA clones from *Hevea brasiliensis*. It is now evident that in *Hevea*, HMGR is encoded by a small gene family consisting of three members. We have previously isc-

Fig. 10. Localization of *hmg1* and *hmg3* mRNA in transverse sections of *Hevea* petiole. A. Dark-field micrograph of section hybridized with the *hmg1*-specific antisense probe. B. Dark-field micrograph of section hybridized with the *hmg3*-specific antisense probe. C and D. Light micrographs of above sections stained with toluidine blue. E. Dark-field micrograph of section hybridized with the *hmg1*-specific sense probe. F. Dark-field micrograph of section hybridized with the *hmg3*-specific sense probe. E, epidermis; R, cortex; P, phloem; L, laticifer; X, xylem; T, pith; C, cambium. Bar represents 100 μm.

lated and described *Hevea hmg1* and *hmg2* [10]. While three genes encode HMGR in *Hevea*, only one class of HMGR is known in mammals [9, 22], two forms occur in yeast [4, 5] and in other plants there have been indications of the presence of two genes in *Arabidopsis* [8] and potato [34] and one gene in tomato [25].

Our results from northern analysis, primer-extension analysis and *in situ* hybridization studies provide evidence that *Hevea hmg* genes are

differentially regulated and therefore likely to perform different functions. We found that *Hevea hmg1* and *hmg3* respond differentially to ethylene. Results from primer-extension analysis indicated that *hmg1* is inducible by ethylene while *hmg3* is constitutively expressed. *Hevea hmg1* and *hmg3* were also found to be expressed differentially. We had earlier shown by northern analysis that *hmg1* is more expressed in laticifer than leaf [10]. We have confirmed these results by primer-extension analysis and have further shown that, in contrast, *hmg3* is expressed in equal amounts in leaf and laticifer. Furthermore, we have shown by *in situ* hybridization studies that *hmg1* is expressed predominantly in the laticifers, the cells specific to rubber biosynthesis, whereas *hmg3* is not cell-type-specific. We propose that *hmg1* is likely to encode the enzyme involved in rubber biosynthesis while *hmg3* is possibly involved in isoprenoid biosynthesis of a housekeeping nature in *Hevea*. Our results suggest that independent isoprenoid pathways do occur and the pathway for rubber biosynthesis in *Hevea* is distinct from the pathway(s) leading to the biosynthesis of other isoprenoid compounds in plants. It thus appears that laticifer-specific *hmg1* which is likely to be involved in rubber biosynthesis is possibly unique to *Hevea* and does not possess a corresponding member in plants which do not produce rubber. In contrast, it is likely that *Hevea hmg2* and *hmg3* correspond to the two *hmg* genes found in other plants, particularly in *Arabidopsis* [8] and in potato [34]. The two potato *hmg* genes have been shown to be differentially regulated. They are either induced by wounding or by pathogen challenge. Therefore, it will be of interest to determine in future which of *Hevea hmg2* and *hmg3* is induced by either of these treatments.

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